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TRANSLATION NO. 443

DATE: 11 May 1963

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

ON THE DEMONSTRATION OF STAPHYLOCOCCUS ENTEROTOXIN

/Following is a translation of an article by Dr. Malte Kienitz and Professor Doctor Rudolf Freuner, Director of the Hygiene Institute of the Hanseatic City of Luebeck, in the German-language periodical Zentralblatt fuer Bakteriologie (Central News for Bacteriology), Vol. 173, 1958, pages 203-212. Submitted on 4 Jun 58./

2. Investigations into the Effect of Enterotoxin-forming Staphylococci on Chick Embryos

/Note: 1. "The Known Biological Procedures" (1. Die bekannten biologischen Verfahren") appeared in the Zbl. F. Bakt. (Central News for Bacteriology), Vol. 168, 1958, pages 117-132./

The incubated hen egg serves in biology, and especially in virus research, as a frequently used test object and recourse has often been had to it for the infection with staphylococci. Green and Birkeland (4), Knothe (6), Jern and Meleney (5), as well as Frappier and Sonea (3), have published interesting findings on this subject. It can be accepted as certain that the chick embryo in the majority of cases dies after injection of a few staphylococci (Micrococcus pyogenes var. aur.) (Lethal dose 50 according to Frappier and Sonea less than 100 germs). However, toward the end of the incubation period, the sensitivity to the infection clearly decreases. The cause of this mortality has by no means been clarified. So far, one may only assume that a part of the toxins or hemolysins formed by the staphylococci has a damaging effect on the chick embryo. This is also supported by the observations of Knothe (6), that the inoculation of hen eggs with a staphylococcus strain without pigment-formation and hemolysis (Micrococcus pyogenes var. albus) does no recognizable damage to the embryos.

We were interested in discovering whether enterotoxin-forming staphylocci can be differentiated in the infection test from those

without enterotoxin production. At the same time, the significance of the diminishing sensitivity of older chick embryos to infection with staphylococci was to be tested. Finally, we intended to observe the behavior of several strains of micrococcus pyogenes var. albus in the infection test.

Material and Methods

Hen Eggs. The eggs, obtained from a chicken breeder, were from sickness-free stock. On the basis of previous experience, we chose the eggs of one-year-old pullets, in each case of a single strain and breed (brown Italian and Leghorns exclusively). As far as possible, pullets of the same weight (C) were used. In part, pre-incubated eggs were used. These were brought to our Institute twenty-four hours before the start of the test. When the incubation was done by us from the first, the age of the eggs never exceeded 48 hours.

Incubation Technique. Old-style electrical flat-surface incubators and (standard) Rhret flat-surface incubators with roller turners were used. Temperature regulation by contact thermometer or ether membrane. Humidity measurement by hair hygrometer. Before they were placed in the incubator, the eggs were freed of coarse dirt (not washed) and marked. Starting temperature 38.5°C (first incubation day). By raising the intrinsic heat of the eggs and using the heat-regulator, an end temperature of 39.5°C was attained (twentieth day). From the fourth up to the eighteenth day of incubation, the eggs were turned twice a day. Moving the eggs was done in the way that is usual among incubator operators. The daily cooling prescribed for the use of flat-surface incubators was superfluous in view of the daily candling check which was conducted from the seventh incubation day with simultaneous elimination of eggs that had died or were misformed (air bubble at the sharp end, etc.). Relative humidity - 50-60 percent, and 70-85 percent at hatching.

Injection Technique. In these tests, we used the allantoic cavity technique. The technique is easily learned and, properly employed, seldom brings about by itself the death of embryos. In order to exclude possibilities of mistakes in this connection, it is recommended that some control series with an injection of 0.1 milliliter of physiological saline solution precede the tests themselves. For the injection, we used tuberculin syringe and cannula No. 20. The hole, which after disinfection (with iodine alcohol) of the shell is made with an electric dental drill (1.5 mm diameter Rosenkopf drill), should be just passable with the cannula. The shell-membrane then remains undamaged. Damage to the shell-skin, small hemorrhages and the like must be recorded. The injection dose of 0.2 milliliter can only

increased after removal of corresponding amounts of the allantoic fluid (2nd drill-hole), otherwise the inner pressure of the egg becomes too high. The unavoidable injury to the egg-membrane in the actual injection itself almost always leads to small hemorrhages in eggs incubated for more than 12 days, but these, in our experience have no influence on the hatching results. Breaks in the shell are sealed with adhesive tape and Zapon varnish; the air bubble is marked and its enlargement is checked daily as the criterion of the optional air humidity in the incubation space.

Judgment of the Changes in the Egg. Only the death of the embryo is to be used as the measure of the effect of the injected cultures. The first candling of the eggs with the test lamp takes place 24 hours after the injection, and is repeated daily until hatching. Often, there occurs a transient or, sometimes persistent swelling of some of the individual peripheral vessels, yet this is no more a sign of incipient death of the egg than is the slackening of the amnionperistaltis (average frequency with ten-days-old embryo -- 16 minutes). If, after long warming over the test lamp there is no movement ascertainable in the egg, a note of this is put in the record. The next check usually reveals sure signs of death in these eggs. We regard as such the extensive involution of the visible vessels and the complete lack of active movements of the embryos that are swimming "free" in the egg. Remains of vessels are not infrequently visible for a rather long time. After the fourteenth incubation day the judgment as to death becomes difficult owing to a diminution of active movements of the embryo in any case. The vessels are now much more difficult to perceive. Here, one has to rely on repeated checks and ones own experience.

Staphylococcus Strains. As test strains, we used staphylococcus strains S-6, 147, 161, 196 and 254 -- recognized as sure exotoxin-formers -- from the Food Research Institute in Chicago, and also strains 360, 127 and 165, isolated from the stool of patients with postantibiotic enterocolitis. Strain 165, like control strain 200, and the coagulase-negative Oxford strain of our Institute, does not produce measurable antitoxin. As a further control, we used 5 strains of micrococcus pyogenes var. albus. All enteritis strains and strain 200 showed: a positive coagulase reaction, fermented Mannitol within 24 hours, and liquefied gelatin in the stab culture in a funnel shape. Further determination was made in each case of hemolysin /Definition and determination of the staphylococcus hemolysins according to the generally-accepted principles, bibliographical list in 7/, pigment formation, sensitivity to seven antibiotics (sulfonamid, erythromycin, penicillin, streptomycin, tetracyclin, chloromycetin, and oleandomycin) in the plate test, and to erythromycin and penicillin again in the tubule dilution test.

The inoculation was -- when not expressly otherwise described -- of cultures that were sixteen hours old and incubated in grape-sugar broth under normal atmospheric conditions at 37°C, with constant maintenance of a dose of 0.1 milliliter. Ascertainment of the number of germs in the individual cultures was not carried out, since this factor -- owing to the swift spreading of the staphylococci in the egg -- does not represent any source of error for the judgment of the findings (3, 8).

Results

The behavior of enterotoxin-forming staphylococci in the infection test with 9½ days-old chick embryos is indicated by Table 1. No significant differences from strains that do not form enterotoxins, i.e., strains 200, 165 and Oxford, could be perceived. Strain 196 offers the most striking result, in that it in contrast to the other strains, produces high titers of β - hemolysin (minimum hemolytic dose 1024) and also produces α - hemolysin. In addition to 196, only No. 165 shows a medium β - hemolysin titer (minimum hemolytic dose 64). The remaining strains form α - hemolysins exclusively.

The infection of 12 and 16 days old chick embryos confirms the observation already made by Knothe (8) that, with the 11th-12th incubation day the sensitivity maximum of the chick embryos to injury by staphylococcus toxins has passed. The differing results in the case of the strains tested are to be seen from Table 1.

Proceeding from the fact that the production of enterotoxin in cultures in a 20-30 percent CO_2 gaseous vapor atmosphere can be considerably intensified [Bibliography, see in 7], we inoculated 9½ days-old chick embryos with cultures that had been incubated for 72 hours with 30 percent CO_2 atmosphere and at 37°C (Table 2).

In addition to the micrococcus pyogenes var. albus 1 (equals staph. alb. 1) from our collection, we tested four additional strains, which did not show either a pigment formation or a positive coagulase reaction, did not ferment Mannitol, and otherwise showed all the characteristics of a micrococcus pyogenes var. albus, but which nonetheless apparently had to be considered as the germs supplying an infection (cystitis, conjunctivitis). In contrast to staph. alb. 1, one found in these strains, in part, a very extensive insensitivity to the seven antibiotics mentioned above. The findings resulting from the infection tests with staph. alb. 1-5 are listed in Table 3.

Table 1
Infection tests on 9½, 12 and 16-day-old chick embryos
(dose: 0.1 ml, 16 hours culture)

Name of strain	α-/β- Hem.-titer (MHD)	Age of eggs in days	Number of eggs	Death at hours after injection 24	48	72	96	>96	Hatched or died Hatching
196(E)*)	512/1024	9½	6	5	1	-	-	-	-
		12	6	2	3	-	-	1	-
		16	6	1	2	-	-	2	1
165(-)	256/64	9½	6	3	3	-	-	-	-
		12	6	1	2	-	1	-	2
		16	6	1	2	1	-	1	1
161(E)	256/-	9½	6	3	1	2	-	-	-
		12	6	1	2	2	-	-	1
		16	6	-	1	-	2	1	-
S-6(E)	128/-	9½	6	4	1	1	-	-	-
		12	6	3	1	2	-	-	-
		16	6	2	2	1	-	1	-
360(E)	64/-	9½	6	4	1	1	-	-	-
		12	6	2	-	2	-	1	1
		16	6	2	1	-	1	-	2
200(-)	64/-	9½	6	3	-	1	-	1	1
		12	6	1	1	2	-	1	1
		16	6	-	2	-	-	1	3
254(E)	32/-	9½	6	1	3	2	-	-	-
		12	6	1	1	-	2	1	1
		16	6	1	1	-	-	1	3
Oxford(-)	32/-	9½	6	3	-	1	1	-	1
		12	6	-	1	2	-	1	2
		16	6	2	2	-	1	1	-

(continued)

(Table 1 continued)

		9½	6	1	-	2	-	1	2
147(E)	16/-	12	6	-	1	-	-	2	3
		16	6	-	-	-	1	1	4
		9½	6	1	1	-	-	3	1
127(E)	8/-	12	6	-	1	-	2	-	3
		16	6	1	-	-	-	1	4

*) (E) = enterotoxin formers

Table 2

Infection tests on 9½ day-old chick embryos
(dose: 0.1 ml, 72 hours and at 30 percent CO_2 incubated culture)

Name of strain	Number of eggs	Death at hours after injection 24	48	72	96	.96	Hatched or died hatching
196(E)*)	6	4	2	-	-	-	-
165(-)	6	4	2	-	-	-	-
161(E)	6	3	3	-	-	-	-
S-6 (E)	6	2	4	-	-	-	-
360(E)	6	1	3	1	-	1	-
200(-)	6	2	3	-	1	-	-
254(E)	6	3	2	1	-	-	-
Oxford (-)	6	2	2	-	1	1	-
147(E)	6	1	1	1	1	1	1
127(E)	6	1	1	-	1	1	2

*) (E) = enterotoxin formers

Table 3
Infection tests with M. pyog. var. alb. on 9½ day-old (above) and on 12 day-old (below) chick embryos (dose: 0.1 ml, 16 hours culture)

Name of strain	Number of eggs	Death at hours after injection 24	48	72	96	More than 96 hrs.	Hatched or died hatching
Staph.alb.1	6	-	-	-	-	-	6
Staph.alb.2	6	-	-	1	-	2	3
Staph.alb.3	6	2	-	1	1	1	1
Staph.alb.4	6	1	4	1	-	-	-
Staph.alb.5	6	-	-	-	1	-	5
Leerbouillon	6	-	-	-	-	-	6
Staph.alb.1	6	-	-	-	1	-	5
Staph.alb.2	6	-	-	-	-	2	4
Staph.alb.3	6	1	-	-	-	1	4
Staph.alb.4	6	1	1	1	-	1	2
Staph.alb.5	6	-	-	-	-	1	5
196 (E)	6	1	3	-	-	1	1
Leerbouillon	10	-	-	-	-	-	10

Finally, we were interested in the behavior of infected embryos after treating with erythromycin. Some strains that had reacted quite variously in the preceding tests -- strains 196, 161, 127, 360, 165 and 200 -- were chosen for these tests. The sensitivity of these strains to erythromycin in the tubule dilution test is noted in Table 4. Following the usual injection of the liquid culture, and after a wait of 10 minutes, erythromycin (Schering "Erycin") in doses of 10 micrograms and 5 micrograms was subsequently injected. The shifting of the death time, and the higher survival rates, can, in contrast to the values obtained in the parallel test with embryos not treated with erythromycin, be seen perfectly (Table 4)

As soon as possible after the established death of the embryos the egg was -- in all the tests mentioned here -- opened and the contents examined. In many cases autolytic changes had made any determinations impossible. Unfortunately, we were also unable to carry out histological examinations. The macroscopic pathological anatomy findings corresponded to those described by Knothe (8). Extended surface hemorrhages, including ones in the parenchymatous organs, could be observed by us in almost every case.

The inoculated strains could be bred from the sterile-removed allantoic fluid. A report is given in the third part of this study (7) on the germs occasionally to be bred out of the yolk and white of hen eggs. It was possible to observe the surviving chicks further. So far, they do not differ in any way from control animals of the same age. Inventory of micro-organisms in the hatching of infected animals was not carried out regularly. The cultures prepared from chicks treated with erythromycin remained sterile with few exceptions.

Discussion of the Results

Demonstration of enterotoxin-forming staphylococci is not possible with the aid of the infection test on incubated hen eggs on the tenth or twelfth or sixteenth incubation day. The death of the infected embryos is evidently not induced by, or not exclusively induced by the staphylococcus enterotoxin. This is indicated particularly by the observation that such enterotoxin-formers as strains 147 and 127 display a less injurious effect than the 200 and Oxford non-enterotoxin forming strains.

Table 4

Infection tests on 9½ days old chick embryos with addition of erythromycin (0.1 ml Erycin = 10 milligrams; 0.5 ml Erycin = 5 milligrams); (E) = enterotoxin formers

[Note: The tests listed here were carried out in the course of the same study as the tests of Table 1. The controls without addition of erythromycin are taken from that table.]

Name of strain	Sensitivity to erythromycin (γ = milligram)	Number of eggs	Dose	Death at hours after injection 24	48	72	96	over 96	Hatched or died hatching
196 (E)	2 γ/ml	6	0.1 ml culture	5	1	-	-	-	-
		3	0.1 ml culture + 0.05 ml Erycin	1	-	-	-	-	2
		3	0.1 ml culture + 0.1 ml Erycin	-	-	-	1	-	2
161 (E)	0.25 γ/ml	6	0.1 ml culture	3	1	2	-	-	-
		3	0.1 ml culture + 0.05 ml Erycin	-	-	-	-	1	?
		3	0.1 ml culture + 0.1 ml Erycin	-	-	-	-	-	3
127 (E)	0.1 γ/ml	6	0.1 ml culture	1	1	-	-	4	-
		3	0.1 ml culture + 0.05 ml Erycin	-	-	-	-	-	3
		3	0.1 ml culture + 0.1 ml Erycin	-	1	-	-	-	2
36c (E)	0.5 γ/ml	6	0.1 ml culture	4	1	1	-	-	-
		3	0.1 ml culture + 0.05 ml Erycin	-	2	-	-	-	1
		3	0.1 ml culture + 0.1 ml Erycin	-	-	-	-	-	3

(continued)

(Table 4 continued)

165 (-)	0.1 γ/ml	6	0.1 ml culture	3	3	-	-	-	-
		3	0.1 ml culture + 0.05ml Erycin	-	-	1	-	-	2
		3	0.1 ml culture + 0.1 ml Erycin	-	1	-	-	-	2
200 (-)	0.5 γ/ml	6	0.1 ml culture	3	-	1	-	1	1
		3	0.1 ml culture + 0.05ml Erycin	-	1	-	-	-	2
		3	0.01ml culture + 0.1 ml Erycin	-	1	-	-	-	2
--	--	10	0.1 ml Erycin	-	-	-	1	-	9

As we received, from Chicago, the five test strains once again in fresh cultures in the Spring of 1958 and used them directly for the test after only one passage. The possibility of degeneration is fully excluded in the case of strain 147. Strain 127 was activated beofre the beginning of the investigations in accordance with the technique employed by Jordan and Burrows (6). However, no final conclusions are possible because while each strain, for example, does produce α- hemolysin this occurs at the same point in time with differing results being found, and so there can also occur some changing titer levels during the growth in the hen egg.

Then again with incubation of the culture in a CO_2 atmosphere, a possible enterotoxin effect is obscured by the other toxins that are simultaneously forming. The effect of the living bacteria and the culture filtrates made from them on chick embryos is not always in our experience identical.

The seasonal fluctuations in the sensitivity of chick embryos to effects from outside -- in particular to injected toxins -- is considered in the discussion of the toxin tests (7). The findings reported here were obtained in the February to April period, the most favorable one for the vitality of chick embryos. The natural mortality rate of embryos not inoculated or else treated with physiological saline solution was, for these months -- including chicks that failed to complete hatching -- barely ten percent counting from the fifth incubation day onwards.

If, among the multiplicity of staphylococcus toxins, one seeks the agent that is pathogenous for chick embryos, then β- hemolysin and coagulase can easily be excluded. β- hemolysin is formed in notable amounts only by strains 196 and 165, from among the strains investigated by us. The nonetheless significantly effective Oxford strain is perfectly coagulase-negative. Frequent attempts to establish the lethal toxin (mouse intraperitoneally, rabbit intravenously) in Oxford filtrates, have so far always failed. At the same time, the α- hemolysin titers (Technique of Determination. See 7,) included in Table 1 do allow certain relationships to be perceived between titer levels and the number of embryos killed by the several strains. Nevertheless α- hemolysin cannot alone be held responsible for the death of the embryos, as is visible from the findings of Table 3.

The behavior of the five strains of micrococcus pyogenes var. albus in the infection test was of special significance for the questions asked in this study. As is known, enterotoxin-forming "white" staphylococci are not infrequently isolated (1, 2, 9) in

cases of food poisoning. But after having observed enterotoxin-forming staphylococci also exhibiting a negative coagulase reaction (11), one has to reckon with the fact that a highly pathogenous enteritis micro-organism is hiding beneath an apparently harmless staphylococcus. The definition of the micrococcus pyogenes var. albus or staphylococcus albus is not consistent. Our routine procedure is to regard as staphylococcus albus the gram-positive cocci which, morphologically, resemble the micrococcus pyogenes var. aureus and which exhibit porcelain-white pigmentation, negative plasma-coagulase, absence of Mannitol fermentation, and absence of hemolysis. Strains 2-5 -- which had been judged from these points of view -- and another strain from our collection (No. 1), were tested at the end of the infection tests, after which the infection of embryos with staph. alb. 1 was carried out as a control, parallel with the preceding experiments; the result was always negative. The death of the embryos in the eggs infected with staph alb. 3 and 4 surprised us. We isolated the strains that had been inoculated, brought them once more into the incubated hen egg after multiple passages of grape-sugar broth on the 7 percent sheep's blood-agar plate (alternating between aerobic and anaerobic incubation) in the technique given by Knothe (8), and repeated this process once more. The result of these rather time-consuming proceedings were meager. The properties of staph. alb. 1, 2 and 5 remained unchanged. Staph. alb. 3 like staph. alb. 4 exhibits a weak Mannitol fermentation after about 50 hours; further, staph. alb. 4 exhibits a α- hemolysis on the seven percent-sheep's blood-agar plate. The coagulase reaction of all strains was consistently negative. The differentiation of coagulase-negative, non-pigment forming, enterotoxin strains from similar ones without enterotoxin effect would be, in our opinion, extremely difficult. On the other hand, we would -- by way of expanding the results of Knothe (8) -- like to regard the infection test on the incubated hen egg as a good criterion of the pathogenicity of the staph. alb. isolated in human infection and, in particular, isolated from otherwise sterile body fluids. Regardless of the pending classification problems, this gives the doctor treating the case an indication of the significance of the micro-organisms that are generally regarded as apathogenous.

It will be the task of further investigations to define more closely so far as possible the components that are deadly to chick embryos; they can evidently be peculiar to both these representatives of the genus micrococcus. The pathogenous effect of the α- hemolysin, of the entero-toxin, and presumably also of other toxins, makes the success of such efforts very questionable to judge by our experiences so far.

With respect to the problem that has been of particular interest to us for a long time -- that of an effective therapy for the post-antibiotic staphylococcal enterocolitis -- we tried the effectiveness in some test series of erythromycin (Schering "Erycin") injected into the egg about 10 minutes after the liquid culture. In spite of the high degree of dilution that the antibiotic undergoes in the egg, it became clear that there was a clear shifting of the death date with simultaneous raising of the survival rate, for the embryos treated. Erythromycin in the doses we used has no perceptible injurious effect on chick embryos. It would be of interest to find out the time period between infection and treatment which still leaves the life-maintaining or life-prolonging effect of the erythromycin perceptible, and whereby, at the same time, the onset of the irreparable injury of infected chick embryos could be determined. The testing of antibiotics in the chick embryos test (5) should count as a good criterion for the effectiveness of the medicine in vivo. [Note: See also the reports of H. Knothe and D. Thon on antibiotics studies (penicillin, streptomycin, tetracyclin) on the incubated hen egg in Arzneimittelforschung (Medicaments Research, 6, 16-21 and 703-712 (1956)].

The tests described here did not bring any usable results as regards a possible demonstration of enterotoxin-forming staphylococci through infection of incubated hen eggs. On the other hand we did find some very interesting points of approach for further experiments, which would aim at an exact analysis of the way in which different staphylococcus toxins affect the chick embryo. In this connection, the titer determination of the filtered, liquid, content of infected eggs -- hitherto only used as a spot check -- would be conducted as routine. We hope further that we shall learn more of the course of death from an electrocardiogram of the embryo that has been derived from a modified procedure of Wertheim-Salomonsen (12). The question of what is the practical value of such investigation for human medicine can hardly be better answered than by the statement of Romanoff and Romanoff (10): "the chick embryo is excellent test material because its tissues are more or less susceptible to the same toxin as human tissues." (page 792).

Summary

The visible effects of the infection of 9½, 12- and 16-day old chick embryos is similar to the picture that has been given so far in the literature on infection with staphylococci without enterotoxin production.

The incubation of the tested strains also in a 30 percent CO_2 atmosphere brought no further results in each test series other than the raising of the death rate.

Micrococcus pyogenes var. albus -- accepted as apathogenous for chick embryos -- can have the same toxic effectiveness as micrococcus pyogenes var. aureus. The infection test with micrococcus pyogenes var. albus on incubated hen eggs should therefore be employed particularly in persistent infections (cystitis, conjunctivitis) and as a pathogenicity test in the demonstration of this microorganism in the fluid or the circulating blood.

On treating infected chick embryos with erythromycin (Schering "Erycin") a clear effect of this antibiotic was perceptible.

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